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**(57) Abstract**

A method for the preparation of cells (useful as biosensors) which present on their surface a mutant of a natural G-protein coupled receptor (GPCR), said mutant being capable of binding a desired ligand which the natural receptor cannot, which method comprises: generating a diverse collection of replicable expression plasmids which comprise (i) a coding sequence coding for a mutated form of a natural GPCR, wherein the mutation is of one or more amino acids in the transmembrane and/or extracellular domains of the natural GPCR, and (ii) expression control sequences operatively linked to said coding sequence to control expression of the mutant GPCR; transforming cells of a host organism with said diverse collection of plasmids; culturing the resultant collection of transformed cells for a sufficient time to permit expression of mutant GPCR from plasmids replicating within such cells; contacting the cultured cells with the desired ligand; and isolating cells wherein the surface presented mutant GPCR expressed by such cells binds to the desired ligand.

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# HOST CELLS EXPRESSING MUTANTS OF A NATURAL G-PROTEIN COUPLED RECEPTOR (GPCR); SACCHAROMYCES CEREVISIAE STE2 GENE, AND THEIR USE AS BIOSENSORS

## Field of the invention

This present invention relates to a method for the preparation, identification and isolation of cells expressing a mutant of a natural G-protein coupled receptor (GPCR) said mutant being capable of binding a desired ligand which the natural receptor cannot, and the use of such cells as biosensors.

## Background to the invention

Biosensors have enormous potential because of their wide range of applications in diagnostics, environmental monitoring, on-line process monitoring and other analytical assays. Biosensors are so called because the sensing element, the component that confers ligand specificity, is biological in origin. The sensing element is usually proteinaceous, typically an enzyme or an antibody, though some applications use complex cell fractions or even whole organisms. Applications using nucleic acid sensors are being developed. The advantages offered by biosensors over conventional analytical techniques are typically specificity, resolution and the possibility of real-time monitoring. In many cases biosensors will also offer improvements in sensitivity and price over existing analytical techniques.

The major problem in biosensor development is the identification of a sensing component with specificity for a desired ligand.

Receptor proteins are nature's biosensors. They are membrane proteins that allow cells to detect molecules in the extracellular environment. A wide range of ligands can be detected ranging from macromolecular proteins through low molecular weight peptides to small organic compounds and metal ions. Receptors can even detect light by using appropriate cofactors such as retinal. One class of natural receptors is the G-protein coupled receptor (GPCR) class. These receptors are recognised from their primary sequence by a distinctive pattern of seven hydrophobic segments which form trans-membrane helices. Ligand binding occurs on the external face of

the receptor, or, for smaller more hydrophobic ligands, within the core of the transmembrane domains. Ligand binding leads to activation of the receptor which in turn triggers intracellular signalling through a cytoplasmic heterotrimeric G-protein complex, which detects changes in the receptor conformation on the cytoplasmic side of the membrane. "The G-protein linked receptor Facts Book", S Watson and S Arkinstall, Academic Press Ltd., provides a general background review of GPCRs.

#### **Brief Description of the Invention.**

In general, receptor/ligand binding interactions are highly sensitive to sequence changes in the binding domain of the receptor molecule. The usual anticipated result of such mutation is reduction or destruction of binding affinity of the natural ligand for the natural receptor (reviewed by Savarese and Fraser in *Biochem J.* 283:1-19, 1992), or, in the case of receptors which respond with different binding affinities to ligands which are members of a recognised class, fortuitous conservative mutation of the natural binding site may produce minor changes in configuration, resulting in a slight shift of the preference of the receptor for one member of the ligand class rather than the natural ligand or a pharmacophore of the natural ligand. Thus there has been little ground for optimism that any given receptor molecule might be randomly mutated, or mutated in the binding domain, to produce a mutant receptor having a completely altered specificity, ie being capable of binding a desired ligand which the natural receptor cannot.

This invention is based on the inventor's finding that receptors of the GPCR type are in fact highly responsive to mutation in the transmembrane and extracellular domains to produce mutant GPCRs capable of binding ligand molecules which are not bound by natural GPCRs. That finding leads to a method for the preparation of cells (useful as biosensors) which present on their surface a mutant of a natural G-protein coupled receptor (GPCR), said mutant being capable of binding a desired ligand which the natural receptor cannot.

**Detailed description of the invention**

According to the invention there is provided a method for the preparation of cells (useful as biosensors) which present on their surface a mutant of a natural G-protein coupled receptor (GPCR), said mutant being capable of binding a desired ligand which the natural receptor cannot, which method comprises:

- (a) generating a diverse collection of replicable expression plasmids which comprise (i) a coding sequence coding for a mutated form of a natural GPCR, wherein the mutation is of one or more amino acids in the transmembrane and/or extracellular domains of the natural GPCR, and (ii) expression control sequences operatively linked to said coding sequence to control expression of the mutant GPCR;
- (b) transforming cells of a host organism with said diverse collection of plasmids;
- (c) culturing the resultant collection of transformed cells for a sufficient time to permit expression of mutant GPCR from plasmids replicating within such cells;
- (d) contacting the thus cultured cells with the desired ligand; and
- (e) isolating cells wherein the surface presented mutant GPCR expressed by such cells binds to the desired ligand.

The host organism cells transformed at step (b) *supra*, may be capable of expressing a recognisable phenotypic marker following signal transduction mediated by ligand binding to mutant GPCR expressed by a plasmid replicating within such cells, and in this case, at step (e) *supra* the cells isolated are those wherein the phenotypic marker is activated by binding of the desired ligand to mutant GPCR expressed in such cells.

The method of the present invention enables the creation and identification of cells presenting mutant GPCRs which bind ligands other than those bound by the natural GPCR from which they are derived. It is preferred that ligand/receptor binding triggers signal transduction which then activates a phenotypic marker, but this is not essential. Binding of the ligand to the receptor can alternatively be detected directly; for example, by exposing to a ligand that has been radiolabelled and detecting the bound ligand using autoradiography. When using an activatable phenotypic marker, the test for whether a given ligand is or is not bound to a GPCR is the appearance or non-appearance of the recognisable phenotypic marker in cells expressing that GPCR and that marker when such cells are contacted with that ligand.

For the purposes of this invention a ligand which binds the natural GPCR with a dissociation constant greater than or equal to 1mM, on average in at least ten separate trials, is not considered capable of binding the natural receptor. This can be most conveniently determined using the Ste2 receptor binding assay described in Clark *et al*, Journal of Biological Chemistry, **269**(12), 8831-8841 (1994), amended to use the desired radiolabelled ligand in place of alpha factor, and using a scatchard plot or other appropriate analytical method to determine the binding constant.

Transformation as used herein, refers to the importation of nucleic acid into the host cell and includes importation into mammalian cells which is often termed transfection in the art.

An important result of the invention is its ability to create and identify cells presenting mutant GPCRs which are capable of binding a ligand which is substantially dissimilar to the ligand or ligands bound by the natural GPCR. Substantial dissimilarity in the present context may be in terms of molecular shape and/or volume, type of bioactivity, pharmacophore, or chemical type.

For step (a) of the method of the invention, standard laboratory methods are known for the generation of a diverse library of mutant genes from a naturally occurring

gene, and these methods may be applied to the generation of the mutant GPCR genes incorporated in the diverse collection of replicable expression plasmids of step (a). Thus random *in vitro* mutagenesis, for example using the method described by Kunkel (Proc. Natl. Acad. Sci. USA **82**:488-492, 1985) with oligonucleotides synthesised to incorporate a percentage of erroneous bases, within the regions coding for the transmembrane or extracellular domains of the natural GPCR gene, or cassette mutagenesis using doped oligonucleotides are known techniques which may be used.

A suitable mutagenesis procedure (adapted from the Kunkel method) is described in Example 1 (*infra*). Essentially, oligonucleotide primers carrying a percentage of randomly misincorporated bases, complementary to the transmembrane and/ or extra cellular domains, apart from the incorporated nucleotide mismatches leading to the mutations, are synthesised and annealed to single stranded plasmid template DNA containing the GPCR gene. Such single stranded template DNA having been prepared as described below, in a *dut<sup>-</sup>, ung<sup>-</sup>* mutant strain of *E. coli*. Such *dut<sup>-</sup>, ung<sup>-</sup>* *E. coli* strain adds uracil codons in place of thymidine, and lacks the uracil repair enzyme. Double stranded plasmid DNA is prepared *in vitro* using a polymerase to successively add the complementary nucleotide to that on the template strand as the polymerase progresses along from the primer. The thus generated diverse population of double stranded plasmid DNAs are transformed into a *dut<sup>+</sup>, ung<sup>+</sup>* *E. coli* host strain wherein the template strand (which contains uracil codons) is destroyed by the uracil repair mechanism, ensuring that the mutated strand of the plasmid is preferentially replicated yielding a higher proportion of *E. coli* containing a mutant GPCR gene. The diverse population of double stranded plasmid DNAs can then be extracted from the *E. coli* and transformed into the desired host organism for expression of the mutant GPCRs.

The mutagenesis conditions used for the creation of the mutant GPCR genes in the plasmid collection may be selected to control the rate of incorporation of a random mutation at any one position in the oligonucleotide primers, and thus the extent of

mutation relative to the natural GPCR gene. Conditions may be selected for creation of genes coding for mutant GPCRs with from 1 or more amino acid changes relative to the natural receptor. The majority of members of the diverse collection of plasmids may, for example have 2, 3, 4, 5, 6, 7, 8 or more mutations within the regions coding for transmembrane and/or extracellular domains, relative to the natural GPCR gene.

DNA sequences encoding natural GPCRs have already been described (see The G-protein linked receptor Facts Book. S Watson and S Arkinstall, Academic Press Ltd.). For example, the *Saccharomyces cerevisiae* STE2 and STE3 genes (Nakayama et al., EMBO J. 4:2643-2648, (1985)), the *Saccharomyces kluyveri* STE2 gene (Marsh and Herskowitz, Proc. Nat. Acad. Sci. USA 85:3855-3859, (1988)), and the protein sequences of numerous olfactory-specific GPCRs (Buck and Axel, Cell. 65:175-187, (1991); see also WO 92/17585). The starting natural GPCR gene sequence for the method of the invention may be one which codes for a yeast mating factor receptor, for example the Ste2 receptor, or an odour (olfactory) receptor.

The library of mutated receptors can be constructed in a plasmid vector that lends itself both to manipulation in *E. coli* to facilitate the mutagenesis, and transformation into the desired host organism. Numerous suitable plasmids are commercially available. Characteristically, such plasmids will contain two selectable marker genes one for selection in *E. coli* (e.g. antibiotic resistance gene) and the other for selection in the host organism for expression of the mutant GPCRs. Thus if the host organism is an auxotrophic yeast lacking a functional gene coding for an essential enzyme necessary for growth, the functional gene and promoter elements allowing its expression can be used as a selectable marker, e.g. *HIS3*, *URA3*, *LEU2*. Both selectable markers are operatively linked to suitable expression control sequences to control expression of the markers. The plasmid will also contain a cloning site for insertion of the gene of interest operatively linked to control elements (including a suitable promoter) to allow expression of the gene product in the desired host and the origin of replication of the filamentous bacteriophage f1. The latter enables single



stranded plasmid template DNA to be prepared from those bacterial cells containing the plasmid upon infection with an appropriate helper phage. Following helper phage infection, the plasmid enters the f1 replication mode and single stranded plasmid template DNA (preferentially of one strand only) is produced and exported from the bacterial cell as an encapsidated virus-like particle. The single stranded template DNA can be extracted from the culture supernatant using standard precipitation and extraction procedures. This single stranded template DNA can then be used in the mutagenesis reaction for generating the diverse population of mutant GPCR genes.

At step (b) of the method of the invention the host organism into which the plasmid collection is transformed will be one capable of supporting replication of the plasmids. Replicable plasmids may be created at step (a) for replication in mammalian and insect cells, in yeast cells including fission yeast, or in bacteria. Yeast is a convenient host organism because it affords a good combination of genetic flexibility and a natural ability to couple to GPCRs. *Saccharomyces cerevisiae* is suitable.

Preferably, the natural GPCR chosen as the starting point for the method of the invention will couple to the host's own signal transduction pathway, though genes for additional elements necessary for signal transduction, such as G protein subunits, can be co-expressed in the host cell using standard recombinant DNA and transformation technology (e.g. see Lefkowitz *et al.* Science **250**: 121-123, (1990)).

During cultivation of the transformed host at step (c) of the method of the invention, a recognisable phenotypic marker may be expressed following signal transduction mediated by ligand binding to mutant GPCR expressed from one of the diverse collection of plasmids replicated in the transformed host cells. The recognisable phenotype may be growth, inhibition of cell division or induction of enzymes giving rise to colour or fluorescence in the presence of suitable substrates. The preferred phenotypic changes are colourimetric (e.g. beta-galactosidase/XGAL), fluorometric (e.g. green fluorescent protein or beta galactosidase/methyl umbelliferyl-GAL),

luminescent (e.g. luciferase/luciferin) or physiological (e.g. changes in pH, oxygen consumption, ion flux). This signal transduction can be readily achieved in yeast where activation of the endogenous G-protein pathway, normally involved in mediating the response to mating factors, leads to arrest of cell-division. This approach can be adapted to give other convenient phenotypic changes. For example, fusion of an amino acid biosynthetic gene to the pheromone response elements in an appropriate auxotrophic mutant background can render growth dependent on activation of the pheromone signalling pathway. This approach has been used to demonstrate functional coupling of the somatostatin receptor in *Saccharomyces* (Price *et al.*, Molecular and Cellular Biology, **15**, 6188-6195, (1995)). Similarly, fusion to an antibiotic resistance gene, such as an aminoglycoside phosphotransferase, renders growth in the presence of the antibiotic G418 dependent on signalling via the Ste2 receptor.

In a preferred embodiment of the invention the *Saccharomyces cerevisiae* pheromone response elements are introduced before the gene for beta-galactosidase, most conveniently by fusing to the pheromone response elements in the *FUS1* promoter. Receptor activation gives rise to induction of beta-galactosidase which is visualised by the blue colouration released by breakdown of the chromogenic substrate X-gal. An alternative phenotypic selection mechanism is to use genes required for growth under the control of the response elements.

Using standard techniques, the phenotypic marker can either be genetically engineered into the host cell so as to be encoded by DNA within the host organism (episomal or chromosomal), or it can be incorporated into the plasmid that contains the mutated GPCR gene. In a preferred embodiment the gene encoding the beta-galactosidase phenotypic marker is coupled to genes encoding the *Saccharomyces cerevisiae* STE2 mating factor response elements and stably integrated into the chromosome of a suitable *Saccharomyces cerevisiae* organism.

After cultivation of the transformed cells at step (c) of the method of the invention, the cells are contacted with the desired ligand to enable isolation at step (d) of cells whose said phenotypic marker is activated by binding of the desired ligand to mutant GPCR expressed in such cells. This screening operation may be carried out by plating the transformed cells out onto agar plates carrying the ligand of choice, at concentrations to allow discrete clonal growth so as to facilitate isolation of the clones expressing the phenotypic marker. Screening of the transformed cells against a pool of relevant ligands enables identification of clones which signal receptor binding to members of that pool. Colonies responding to the ligand(s) are purified by clonal isolation, using the phenotypic response to identify clones with the desired specificity.

In a further aspect of the invention, the method provides for the additional step of isolating the mutant GPCR from the isolated cells prepared according to the methods of the invention. This may be facilitated by the presence of the haemagglutinin (HA) epitope tag on the C-terminus of the Ste2 protein encoded by pML4, which allows for its immunoaffinity purification from membranes solubilised with appropriate detergents (Root *et al.*, J. Cell. Biol. **188**:95 (1992)). For a discussion of approaches to the purification of GPCRs see Grisshammer *et al.*, Biochemical Journal, **295**, 571-576, (1994).

The gene coding for a mutant receptor with specificity for a desired ligand can be isolated from the cells expressing the mutant receptor using standard techniques available to the skilled person. The gene can be obtained either from isolated plasmid DNA, or if the gene has integrated into the host chromosomal DNA by restriction digestion and subsequent plasmid cloning or by polymerase chain reaction. Thus the invention includes nucleic acid coding for a mutant GPCR from cells prepared according to the method of the invention.

According to a further aspect of the invention there is provided a diverse library of mutant GPCR-presenting cells. This diverse library may exist within a larger library

of cells that present on their surface a natural GPCR.

According to a further aspect of the invention there is provided a diverse library of cells presenting on their surfaces mutants of a natural GPCR said mutants having a specificity for a ligand for which the natural GPCR does not.

In order to identify cells expressing mutant receptors with enhanced affinity, the transformed cells are plated on successively lower concentrations of ligand so as to identify those clones that exhibit the relevant phenotypic changes at lower concentration than the first generation clones. An alternative approach is to use a host strain in which the phenotypic response to receptor signalling is expression of a gene required for cell growth. In this case, cells expressing the mutant receptors are plated on selective media at decreasing ligand concentration and those cells expressing higher affinity receptors will be able to grow at lower concentrations of ligand.

Preferably the mutant GPCR-presenting cells isolated and prepared according to the invention will have a strong receptor/ ligand binding affinity, for example of between  $10^{-12}$  to  $10^{-6}$ M, preferably between  $10^{-12}$  and  $10^{-7}$ M. If it is desirable to increase the binding affinity of positive responding clones identified and isolated at step (e) of the method of the invention, the procedure (a) - (e) may be iteratively repeated. The invention therefore encompasses an iterative application of mutagenesis and screening until the required affinity for the specific ligand is obtained. This is done by re-mutagenesis of the mutated GPCR gene sequence isolated from the isolated cells and creating a second library of diverse plasmids, expressing the second round plasmids in a host organism and isolating the responsive cells as before. The invention therefore includes the additional step of isolating from the cells isolated during step (e) the nucleic acid sequence coding for the said mutant GPCR, and repeating steps (a) - (e) using that sequence as the coding sequence referred to in step (a). This mutagenesis and reselection process can be repeated until a receptor with the desired properties is identified.

Various additional means for enhancing the sensitivity of a cell expressing a GPCR to its specific ligand exist. For example, truncation of the intracellular C-terminus of the yeast mating factor  $\alpha$  receptor gives rise to a 20-fold increase in sensitivity (Boone *et al.*, Proc. Natl. Acad. Sci. USA, **90**, 9921-9925 (1993)). Mutations in the third intracellular loop between transmembrane domains 5 and 6 can give rise to a similar 20-fold increase in sensitivity, and combining these with the C-terminal truncation gives a combined effect of a 400-fold increase in sensitivity (*ibid.*). Similar effects have been seen with other GPCRs such as the yeast  $\alpha$  mating factor receptor (Clark *et al.*, J. Biol. Chem., **269**, 8831-8841 (1994)). Such approaches can therefore be used to supplement the sensitivity of the mutated GPCRs accessible by the invention. Other approaches include elevating receptor expression, and mutating regions of the receptor involved in receptor desensitisation and receptor recycling.

#### Description of the figures

Figure 1 Map of the plasmid vector pML4.

The following examples illustrate the invention.

#### Example 1 Novel receptors based on Ste2, the *S.cerevisiae* $\alpha$ mating factor receptor

##### Construction of the Ste2 expression vector

The STE2 gene for the  $\alpha$  mating factor receptor has been described previously (Nakayama *et al.*, EMBO J., **4**, 2643-2648 (1985). Genbank accession number X03010). A 1548bp *HindIII*-*BamHI* fragment encompassing the promoter (but not the putative  $\alpha$ -2 protein binding site) and the full coding sequence for the STE2 gene product along with a short C-terminal linker sequence encoding in part an epitope from influenza haemagglutinin (HA) was cloned into the yeast/ *E.coli* shuttle vector pYES2 (Invitrogen Inc.), between the *HindIII* and *BamHI* site in the polylinker. This results in the Ste2/HA open reading frame continuing for a further 26 amino

acids into the polylinker of pYES2 (SEQ ID 1). The resultant plasmid carrying STE2 cloned into pYES2 is designated pML4 (see Fig. 1). This plasmid was transformed into an *E. coli* host strain HW87 to create HW87(pML4) which was deposited at the National Collections of Industrial and Marine Bacteria (NCIMB) Ltd., 23 St Machar Drive, Aberdeen, AB2 1RY: Deposit number 40793, 20th March 1996.

## SEQ ID 1

Sequence of the 1548bp *HindIII* to *BamHI* fragment encompassing the *Ste2* gene in pML4. The amino acid sequences of the predicted transmembrane domains are shown in bold type. The *BamHI* junction in pYES2 is also shown. The *Ste2* open reading frame continues for a further 26 amino acid residues into the pYES2 polylinker.

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      10              30              50
AAGCTTTCCTACATATTCAAGATTTTCTGTGGGTGGAATACTATTAAAGGAGTGCTA
TTCGAAAGGATGTATAAGTTCCTAAAAAAGACCCACCTTATGATAAACTCCTCAGCAT
HindIII

      70              90              110
TTAGTATCTTATTTGACTTCAAAGCAATACGATACCTTTTCTTTTACCTGCTCTGGCTA
AATCATAGAATAAACTGAAGTTTCGTTATGCTATGGAAAAGAAAAGTGACGAGACCGAT

      130              150              170
TAATTATAATTGGTTACTTAAAAATGCACCGTTAAGAACCATATCCAAGAATCAAAAATG
ATTAAATATAACCAATGAATTTTACGTGGCAATCTTGGTATAGGTTCTAGTTTTTAC

                                         M
      190              210              230
S D A A P S L S N L F Y D P T Y N P G Q
TCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAA
AGACTACGCGAGGAAGTAACTCGTTAGATAAAATACTAGGTTGCTATTAGGACCAAGTT

      250              270              290
S T I N Y T S I Y G N G S T I T F D E L
AGCACCATTAAC TACCTTCCATATATGGGAATGGATCTACCATCACTTCGATGAGTTG
TCGTGGTAATTGATGTGAAGGTATATACCTTACCTAGATGGTAGGAAGACTACTCAAC

      310              330              350
Q G L V N S T V T Q A I M F G V R C G A
CAAGGTTTAGTTAACTAGTACTGTTACTCAGGCCATTATGTTTGGTGTGAGATGTGGTGCA
GTTCCAAACTCAATTGTCTATGACAATGAGTCCGTAATACAAACCAAGCTACACACAGT

      370              390              410
A A L T L I V M W M T S R S R K T P I F
GCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAACGCGGATTTTC
CGAGCAAACTGAAACTAACAGTACACCTACTGTAGCTCTTCGTCCTTTTTCGGCTAAAAAG

      430              450              470
I I N Q V S L F L I I L H S A L Y F K Y
ATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATCTTCTGACTCTATTTTAAATAT
TAATAGTTGGTTCAAAGTAAACAAAAATTAGTAAACGTAAGACGTGAGATAAAAATTATA

      490              510              530
L L S N Y S S V T Y A L T G F P Q F I S
TTACTGTCTAATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCTCAGTTCATCAGT
AATGACAGATTAAATGAGAAGTCACTGAATGCGAGAGTGGCTAAAGGAGTCAAGTAGTCA

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550 570 590  
R G D V H V Y G A T N I I Q V L L V A S  
AGAGGTGACGTTTCATGTTTATGGTGTACAAATATAATTCAGTCTTCTTGTGGCTTC  
TCTCCACTGCAAGTACAAATACCACGATGTTTATATTAAAGTTCAGGAAGAACCACCGAAGA

610 630 650  
I E T S L V F Q I K V I F T G D N F K R  
ATTGAGACTTCACTGGTGTTCAGATAAAAGTTATTTTCACAGGCGACAACCTTCAAAGG  
TAACTCTGAAGTGACCACAAAGTCTATTTTCAATAAAAGTGTCGCGTGTGAAGTTTTC

670 690 710  
I G L M L T S I S F T L G I A T V T M Y  
ATAGGCCTGATGCTGACGTCGATATCTTTCACCTTAGGGATTGCTACAGTTACCATGTAT  
TATCCAAACTACGACTGCAGCTATAGAAAGTGAATCCCTAACGATGTCAATGGTACATA  
StuI

730 750 770  
F V S A V K G M I V T Y N D V S A T Q D  
TTTGTAAAGCGCTGTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCAAGAT  
AAACATTTCGCGACAATTTCCTACTAACACTGAATATTACTACAAATCACGGTGGGTCTTA

790 810 830  
K Y F N A S T I L L A S S I N F M S F V  
AAATACTTCAATGCATCCAAATTTTACTTGCATCCCTCAATAAACTTTATGTCAATTGTC  
TTTATGAAGTTACGTAGGTGTTAAATGAACGTAGGAGTTATTTGAAATACAGTAAACAG

850 870 890  
L V V K L I L A I R S R R F L G L K Q F  
CTGGTAGTTAAATTGAATTTAGCTATTAGATCAAGAAGATTCCCTGGTCTCAAGCAGTTC  
GACCATCAATTTAACTAAAATCGATAATCTAGTTCTTCTAAGGAACCAGAGTTCGTCAGG

910 930 950  
D S F H I L L I M S C Q S L L V P S I I  
GATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTCCATCGATAATA  
CTATCAAGGTATATAAATGAGTATTACAGTACAGTTAGAAACAACCAAGGTAGCTATTAT

970 990 1010  
F I L A Y S L K P N Q G T D V L T T V A  
TTCATCCTCGCATACAGTTTGAACCAAACCAAGGAAACAGATGTCTTGACTACTGTTGCA  
AAGTAGGAGCGTATGTCAAACCTTGGTTGGTCCCTTGCTACAGAACTGATGACAACGT

1030 1050 1070  
T L L A V L S L P L S S M W A T A A N N  
ACATTACTTGCTGTATTGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAAT  
TGTAATGAACGACATAACAGAAATGGTAATAGTAGTTACACCCGGTGCCGACGATTATTA

1090 1110 1130  
A S K T N T I T S D F T T S T D R F Y P  
GCATCCAAAACAACACAATTACTTCAGACTTTACAACATCCACAGATAGGTTTATCCA  
CGTAGGTTTGTGTTTAAATGAAGCTGAAATGTTGTAGGTGTCTATCCAAATAGGT

1150 1170 1190  
G T L S S F Q T D S I N N D A K S S L R  
GGCAGCGTGCTAGCTTTCAAACCTGATAGTATCAACAACGATGCTAAAAGCAGTCTCAGA  
CCGTGCGACAGATCGAAAGTTGACTATCATAGTTGTGTCTACGATTTTCGTGACAGTCT



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1210          1230          1250
S R L Y D L Y P R R K E T T S D K H S E
AGTAGATTATATGACCTATATCCTAGAAGGAAGGAAACAACATCGGATAAACATTTCGGAA
TCATCTAANTACTAGGATATAGGATCTTCCTTCCTTTGTTAGCCTATTGTGAAGCCTT

1270          1290          1310
R T F V S E T A D D I E K N Q F Y Q L P
AGAACCTTTTGTCTGAGACTGCAGATGATATAGAGAAAAATCAGTTTATCAGTTGCC
TCTTGAAAAACAAGACTCTGACGTCTACTATATCTCTTTTAGTCAAAATAGTCAACGGG

1330          1350          1370
T P T S S K N T R I G P F A D A S Y K E
ACACCTACGAGTTCAAAAAATACTAGGATAGGACCGTTTGTCTGATGCAAGTTACAAGAG
TGTGGATGCTCAAGTTTTTTATGATCCTATCCTGGCAAACGACTACGTTCATGTTTCTC

1390          1410          1430
G E V E P V D M Y T P D T A A D E E A R
GGAGAAGTTGAACCCGTCGACATGTACACTCCCGATACGGCAGCTGATGAGGAAGCAGA
CCTCTTCAACTTGGGCGAGCTGTACATGTGAGGGCTATGCCGTCGACTACTCCTTCGCTCT

1450          1470          1490
K F W T E D N N N L C G R I F Y P Y D V
AAGTCTCGGACTGAAGATAATAATAATTTATGCGGCCGCATCTTTTACCCATACGATGTT
TTCAAGACCTGACTTCTATTATTATTAATACGCCGCCGTAGAAAAATGGGTATGTACAA

1510          1530          1550
P D Y A G Y P Y D V P D Y A G S T S N G
CCTGACTATGCGGGCTATCCCTATGACGTCCCGACTATGCAGGATCCACTAGTAACGGC
GGACTGATACGCCCGATAGGGATAGTCCAGGGCCTGATACGTCTCTAGGTGATCATTCGCC

BamHI pYES2->

1570          1590          1600
R Q C A G I L Q I S I T L A A A R A C I
CGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATC
GCGGTCACACGACCTTAAGACGCTTATAGGTAGTGTGACCCGCCGCGAGCTCGTACGTAG

*
TAG
ATC

```

A library of mutant STE2 receptors can be generated using the pML4 plasmid as a template for the mutagenesis. The presence of the haemagglutinin (HA) epitope tag on the C-terminus of the Ste2 protein encoded by pML4, would enable the mutant GPCR to be isolated from the cells, using for example immunoaffinity purification from membranes solubilised with appropriate detergents (Root *et al.*, J. Cell. Biol. **188:95** (1992)). The presence of the haemagglutinin epitope is however, not necessary for carrying out the invention.

To create a *STE2* expression vector that contained none of the *STE2* promoter region and removed the influenza haemagglutinin epitope, site directed mutagenesis was performed as described below, using single stranded DNA from pML4 and the following oligonucleotide primers.

*STE2* 5' end mutagenesis primer

5'CACTATAGGGAATATTAAGCTTCCAAGAATCAAAAATGTC3' (SEQ ID 2)

*STE2* 3' end mutagenesis primer

5'GGACTGAAGATAATAATAATTTATGAGGATCCACTAGTAA3' (SEQ ID 3)

#### Preparation of single-stranded template DNA and mutagenesis.

The plasmid pML4 was transformed into *E. coli* strain RZ1032 (HfrKL16PO/45 *lysA961-62, dut1 ung1 thi1 recA Zbd-279::Tn10 supE44*) selecting for ampicillin resistance and single stranded DNA prepared by superinfection with phage fd using established techniques. This host gives rise to template DNA with a high percentage of misincorporated uracil. This leads to preferential degradation of the parental strand following transfection of the mutagenesis reaction mixture, and increases the yield of mutants (Kunkel *et al.* Proc. Natl. Acad. Sci. USA **82**:488-492, (1985)).

The mutagenesis was performed essentially as described by Kunkel *et al.* (1987). The mutagenesis primers (25pmole of each) were 5' phosphorylated using polynucleotide kinase and then added to 10µg single stranded pML4 template DNA in a final reaction mix of 100µl containing 70mM Tris pH8, 10mM MgCl<sub>2</sub>. The primers were annealed by heating the mutagenesis mixture at 70°C for 3min followed by 37°C for 30min. The annealed mixture was placed on ice and the following reagents added: 50µl 10 x HM (200mM HEPES, 100mM MgCl<sub>2</sub> pH7.6), 50µl of ATP (10mM), 50µl of all four dNTPs (each at 5mM), 50µl DTT (1mM), 20µl of T4 DNA ligase (1000u), 10µl Klenow fragment of DNA polymerase and water to a

final reaction volume of 500µl. The reaction mixture was then incubated at 4°C for 30 min and then 15°C for 16hr. After the reaction was complete, 500µl of TE (10mM Tris, 1mM EDTA pH8.0) was added.

The mutagenesis reaction mixture was transfected into *E. coli* strain TOP10F' (F'*{lacIqTn10(Tet<sup>R</sup>)}*) *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80/*lacZM15* Δ*lacX74* *deoR* *recA1* *araD139*Δ(*ara-leu*)7697 *galU* *galK* *rpsL* *endA1* *nupG* (Invitrogen), selecting for ampicillin resistance. Twelve ampicillin resistant clones were isolated and plasmid DNA purified using the Qiagen™ kit (Hybaid Ltd) as recommended by the supplier.

Plasmids were screened for the presence of the expected a 1.3kb product by digestion with *HindIII* and *BamHI*. The correct sequence was then confirmed by DNA sequencing of the STE2 gene (SEQ ID 4). The resultant plasmid was designated pHG1.

18

SEQ ID 4

Sequence of the 1321bp *HindIII* to *BamHI* fragment encompassing the *STE2* gene in pHG1. The DNA sequences corresponding to the mutagenesis primers are underlined. The *BamHI* and *HindIII* junctions in pYES2 are shown.

```

      10                      30                      50
HindIII      M S D A A P S L S N L F Y D
AAGCTTCCAGAATCAAAAATGTCGATGCGGCTCCTTCATTGAGCAATCTATTTTATGA
TTCGAAGGTTCTTAGTTTTTACAGACTACGCCGAGGAAGTAACTCGTTAGATAAAATACT

      70                      90                      110
P T Y N P G Q S T I N Y T S I Y G N G S
TCCAACGTATAATCCTGGTCAAAGCACCATTAACACACTTCCATATATGGGAATGGATC
AGGTTGCATATTAGGACCAGTTTCGTGGTAATTGATGTGAAGGTATATACCCCTTACCTAG

      130                      150                      170
T I T F D E L Q G L V N S T V T Q A I M
TACCATCACTTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGCCATTAT
ATGGTAGTGAAGACTACTCAACGTTCCAAATCAATTGTCATGACAAATGAGTCCGGTAATA

      190                      210                      230
F G V R C G A A A L T L I V M W M T S R
GTTTGTTGTCAGATGTGGTGACGCTGCTTTGACTTTGATTGTGATGGATGACATCGAG
CAAAACCAAGTCTACACCACGCTGACGAACTGAACTAAACAGTACACCTACTGTAGTCT

      250                      270                      290
S R K T P I F I I N Q V S L F L I I L H
AAGCAGAAAAACGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGGC
TTGCTCTTTTTTGCGGCTAAAAGTAATAGTTGGTCAAAGTAAACAAAAATTAGTAAAAAGCT

      310                      330                      350
S A L Y F K Y L L S N Y S S V T Y A L T
TTCGCACTCTATTTTAAATTTTACTGCTAATTACTCTTCAGTGACTTACGCTCTCAC
AAGACGTGAGATAAAAATTATAAATGACAGATTAAATGAGAAGTCACTGAATGCGAGAGTG

      370                      390                      410
G F P Q F I S R G D V H V Y G A T N I I
CGGATTTCCCTCAGTTCATCAGTAGAGTGACGTTTCATGTTTATGTTGCTACAAATATAT
GCCTAAAGGAGTCAAGTAGTCATCTCCACTGCAAGTACAAATACCAAGTATGTTATATTA

      430                      450                      470
Q V L L V A S I E T S L V F Q I K V I F
TCAAGTCCTTCTGTGGCTTCTATTGAGACTTCAGTGTGTTTCAGATAAAAGTTATTTT
AGTTTCAGGAAGAACCCGAAGATAACTCTGAAGTGACCACAAAGTCTATTTTCAATAAAA

      490                      510                      530
T G D N F K R I G L M L T S I S F T L G
CACAGGCGCAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACCTTTAGG
GTGTCGCTGTTGAAGTTTTCCTATCCAACTACGACTGCAGCTATAGAAGTGAAATCC

      550                      570                      590
I A T V T M Y F V S A V K G M I V T Y N
```

GATTGCTACAGTTACCATGTATTTTTGAAGCGCTGTTAAAGGTATGATTGTGACTTATAA  
CTAACGATGTCAATGGTACATAAAACATTTCGCGACAATTTCCATACTAACACTGAATATT

610 630 650  
D V S A T Q D K Y F N A S T I L L A S S  
TGATGTTAGTGCCACCCCAAGATAAATACTTCAATGCATCCACAATTTTACTTGCATCCTC  
ACTACATACACGGTGGGTTCTATTTATGAAGTTACGTAGGTGTTAAATGAACGTAGGAG

670 690 710  
I N F M S F V L V V K L I L A I R S R R  
AATAAACTTTATGTCATTTGTCCTGGTAGTTAAATGATTTTAGCTATTAGATCAAGAAG  
TTATTTGAAATACAGTAACAGGACCATCAATTTAACTAAATCGATAATCTAGTTCCTTC  
730 750 770  
F L G L K Q P D S F H I L L I M S C Q S  
ATTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAACT  
TAAGGAACCAAGAGTTCGTCAAGCTATCAAAGGTATAAAATGAGTATTACAGTACAGTTAG

790 810 830  
L L V P S I I F I L A Y S L K P N Q G T  
TTTGTGTTCCATCGATAATATTCATCTCGCATACAGTTTGAAACCAACACAGGGAAC  
AAACAACCAAGGTAGCTATTATAAGTAGGAGCGTATGTCAAACCTTTGGTTTGGTCCCTTG

850 870 890  
D V L T T V A T L L A V L S L P L S S M  
AGATGCTCTGACTACTGTTGCAACATTACTTGGTGTATTGTCTTTACCATTATCATCAAT  
TCTACAGAAGTGTGACACCGTTGTATGAACGACATAACAGAAATGGTAATAGTAGTTA

910 930 950  
W A T A A N N A S K T N T I T S D F T T  
GTGGCCACGGCTGCTAATAATGCATCCAAAACAAACACAATTACTTCAGACTTTACAAC  
CACCCGGTGCCGACGATTATTACGTAGGTTTTGTTTTGTGTTAATGAAGTCTGAAATGTTG

970 990 1010  
S T D R F Y P G T L S S F Q T D S I N N  
ATCCACAGATAGGTTTTATCCAGGCACGTGCTAGCTTTCAAACATGATAGTATCAACAA  
TAGTGTCTATCCAAAATAGGTCGGTGCACAGATCGAAAGTTTGACTATCATAGTTGTT

1030 1050 1070  
D A K S S L R S R L Y D L Y P R R K E T  
CGATGCTAAAAGCAGTCTCAGAAGTAGATTATAGACCTATATCCTAGAAGGAAGGAAC  
GCTACAGATTTTCGTAGAGTCTTCATCTAATATACGTGATATAGSATTCTCCTTCCTTTG

1090 1110 1130  
T S D K H S E R T F V S E T A D D I E K  
AACATCGGATAAACATTCCGAAAGAACTTTTGTCTGAGACTGCAGATGATATAGAGAA  
TTGTAGCCTATTGTGAAGCCTTTCTTGAAACAAAGACTCTGACGTCTACTATATCTCTT

1150 1170 1190  
N Q F Y Q L P T P T S S K N T R I G P F  
AAATCAGTTTTATCAGTTGCCACACCTACGAGTTCAAAAAATCTAGGATAGGACCGTT  
TTTAGTCAAAATAGTCAACGGGTGTGGATGCTCAAGTTTTTTATGATCCTATCTCGGCAA  
1210 1230 1250  
A D A S Y K E G E V E P V D M Y T P D T  
TGCTGATGCAAGTTACAAAGAGGGAGAAGTTGAACCCGTGCACATGTACACTCCCAGATAC  
ACGACTACGTTCAATGTTTCTCCCTCTTCACTTGGGACGCTGATACGTGAGGGCTATG

```

      1270              1290              1310
      A A D E E A R K F W T E D N N N L * BamHI
GGCAGCTGATGAGGAAGCCAGAAAGTTCTGGACTGAAGATAATAATAATTATGAGGATCC
CCGTCGACTACTCCTTCGGTCTTCAAGACCTGACTTCTATTATTATTAATACTCCTAGG

```

This plasmid simplifies manipulation of the gene in *E. coli*, facilitates generation of single strand DNA for mutagenesis by virtue of its phage f1 origin, and allows plasmid maintenance and expression in yeast from an inducible *GAL1* promoter on the vector.

Creating the library of STE2 mutants by local random mutagenesis.

pHG1 was transformed into *E. coli* strain RZ1032 as described above, and single stranded DNA isolated. The single stranded pHG1 DNA was subjected to localised mutagenesis using synthetic oligonucleotide primers. The primers had been synthesised so that at certain positions, the coupling reaction was contaminated with 0.8% v/v of each of the incorrect phosphoramidites, giving an approximately 2.4% probability of incorporation of the incorrect base. This doping was avoided at the 5' and 3' ends of the oligonucleotides, and at base positions which could give rise to a nonsense mutation. The following nine primers, corresponding to the outer half of transmembrane domains 1-7 (TM1-7; see SEQ ID 1), were synthesised according to these principles (R & D Systems Ltd). Lower case indicates bases that, on synthesis, were subjected to contamination by each of the other three phosphoramidites.

TM1:5'CTGTTactCa ggccattatg ttggtgtcA gatgTggtGC AGC 3' (SEQ ID 5)

TM2/2:5'CATTGtttTaatacatttTgcattCT 3' (SEQ ID 6)

TM2/3:5' GCACtctaTtttAaataTtTaCTGTC (SEQ ID 7)

TM3/2:5'GTGCtacaataataattCaagtCCTT 3' (SEQ ID 8)

TM3/3.5'CTTGtggtcttattGaGacttCACTG 3' (SEQ ID 9)

TM4:5'CCATGtaTtt tgtaagcgct gttAaaggta tgattGTGAC 3' (SEQ ID 10)

TM5:5'CTTCAATgca tccacaattt Tacttgcatc ctCaataAAC 3' (SEQ ID 11)

TM6:5'GTTGGttcca tCgataatat tcatcctcgc ataCaGTTTG 3' (SEQ ID 12)

TM7:5'CAGATgtctT gactactgtt gcaacatTac ttgctgATTG 3' (SEQ ID 13)

The mutagenesis was performed as described above. Twelve ampicillin resistant clones were isolated and plasmid DNA purified for DNA sequence analysis using the Qiagen™ kit (Hybaid Ltd) as recommended by the supplier. Sequencing was carried out using a Sequenase™ kit (US Biochemicals) as recommended by the supplier and a sequencing primer which anneals 5' to the region encoding the first transmembrane helix (5'-ggatctaccatcactttcg-3' - SEQ ID 14). This confirmed that an appropriate level of mutagenesis had been obtained (>50% of sequences carrying mutation(s) giving rise to at least one amino acid substitution).

The remaining ampicillin resistant clones (in excess of 10<sup>6</sup>) were pooled by washing the colonies off the plates with 210 mls TY broth. The pooled mutant library was mixed thoroughly and 70 mls stored in 50% glycerol (v/v) at -70°C.

The remaining 140mls of the library was used to prepare plasmid DNA using a scaled up Qiagen™ protocol and the purified plasmid DNA library stored at -70°C until required.

**Example 2 Screening the mutant Ste2 library for new specificities**

The mutant Ste2 library was screened for receptors capable of responding to ligands of interest by transforming the reporter strain ERSC1 (*MATa*, *ste2-10::LEU2*, *FUS1-lacZ*, *cry1<sup>R</sup>*, *ade2*, *his4*, *lys2*, *trp1*, *tyr1*, *SUP4-3am<sup>ts</sup>*, *leu2*, *ura3*). This strain has been deposited at the NCIMB: Deposit number 40792, 20th March 1996. The *FUS1-lacZ* fusion results in  $\beta$ -galactosidase expression in response to activation of the  $\alpha$  factor signal transduction pathway. The *LEU2* insertion in *STE2* inactivates the host receptor so that  $\beta$ -galactosidase expression is dependent on signalling through the mutated receptor transformed into the host.

Plasmid DNA (100 $\mu$ g) comprising the mutated *STE2* receptor library in pHG1 was transformed into ERSC1 using the YEASTMAKER™ Yeast Transformation system (Clontech Laboratories, Inc, Palo Alto, CA) and using the manufacture's instructions for the library scale transformation. The cells were plated on agar plates (2% agar, 2% glucose, 0.67% amino acid free yeast nitrogen base) supplemented with L-isoleucine 30 $\mu$ g/ml, L-valine 150 $\mu$ g/ml, L-arginine 20 $\mu$ g/ml, L-histidine 20 $\mu$ g/ml, L-leucine 100 $\mu$ g/ml, L-lysine 30 $\mu$ g/ml, L-methionine 20 $\mu$ g/ml, L-phenylalanine 50 $\mu$ g/ml, L-threonine 200 $\mu$ g/ml, L-tryptophan 20  $\mu$ g/ml, L-tyrosine 30 $\mu$ g/ml, adenine 20 $\mu$ g/ml but lacking uracil to select for transformants carrying the pHG1 plasmid, which carries a functional *URA3* gene. Incubation was at 30°C for 2-3 days until Ura<sup>r</sup> transformants formed colonies about 1mm in diameter. This scale of transformation yields 10<sup>6</sup>-10<sup>7</sup> transformants. The transformed yeast were pooled by washing the colonies off the plates with YPD broth. The pooled mutant library was mixed thoroughly and stored in 25% glycerol (v/v) at -70°C.

The yeast mutant library was diluted with distilled water and plated on 140mm diameter SC gal/raf agar plates (2% agar, 2%w/v galactose, 1%w/v raffinose, 0.67% amino acid free yeast nitrogen base) supplemented with L-isoleucine 30 $\mu$ g/ml, L-valine 150 $\mu$ g/ml, L-arginine 20 $\mu$ g/ml, L-histidine 20 $\mu$ g/ml, L-leucine 100 $\mu$ g/ml, L-lysine 30 $\mu$ g/ml, L-methionine 20 $\mu$ g/ml, L-phenylalanine 50 $\mu$ g/ml, L-



threonine 200µg/ml, L-tryptophan 20 µg/ml, L-tyrosine 30µg/ml, adenine 20µg/ml but lacking uracil. Incubation was at 30°C for 2-3 days until Ura<sup>+</sup> colonies appeared. The Ura<sup>+</sup> colonies were screened for new receptor specificities by overlaying the plates with 20ml indicator agar. X-GAL indicator agar was prepared as described in Methods in Yeast Genetics, M. Rose, F. Winston, P. Hieter, Cold Spring Harbor Laboratory Press, New York, 1990. With the exception that agar was at 1%w/v, and 2%w/v galactose and 1%w/v raffinose were used as carbon sources. Particular care was taken to ensure the top agar maintained a neutral pH to ensure the X-GAL indicator functioned. The ligand mix was added to a concentration of 200ng/ml per ligand. The components of the ligand cocktail are shown in Table 1. This mixture was diluted 1/100 into top agar to give a final concentration of 200ng/ml per ligand. Plates were incubated at 30°C for 5 days and monitored daily. During this period, colonies which became blue were presumed to be expressing  $\beta$ -galactosidase in response to a ligand(s) present in the ligand cocktail. Positive colonies were picked and grown on paired SC gal/raf plates (lacking uracil) and overlayed with X-GAL indicator medium in the presence and absence of the ligand cocktail. This permitted identification of colonies in which  $\beta$ -galactosidase expression was truly dependent on the ligand cocktail and elimination of surviving constitutive responders.

The identity of the ligand responsible for inducing  $\beta$ -galactosidase expression in each of the responding colonies was determined by replica plating onto six SC gal/raf plates. Following growth of the yeast at 30°C each plate was overlayed with a subset of the ligand cocktail such that the pattern of response for a given mutant strain is diagnostic for the ligand inducing the response. The components of the cocktail were numbered 1 to 63, and assigned to 6 sub-mixtures (SM1, SM2, SM4, SM8, SM16 and SM32) so that the sum of the assigned sub-mixtures equalled the substance identifier number.

This approach permits the identification of strains carrying mutant *ste2* receptors capable of responding to ligands unrelated to alpha factor.

Table 1 Contents of ligand cocktail

No.	Substance	Sigma Cat No.	Concn.	50ml DMSO 50ml Mix	Submixtures					
					SM1	SM2	SM4	SM8	SM16	SM32
1	ampicillin	A9518	-	100mg 0.5ml						
2	tetracycline	T3383	-	100mg 0.5ml						
3	kanamycin	K4000	-	100mg 0.5ml						
4	streptomycin	S6501	-	100mg 0.5ml						
5	chloramphenicol	C0378	-	100mg 0.5ml						
6	glyphosate	P9556	-	100mg 0.5ml						
7	halogenated volatiles	38,483-6	0.1mg/ml	neat 1ml						
8	ethers and phthalates	E1393	2mg/ml	neat 0.5ml						
9	phenols	4-7899	0.5mg/ml	neat 2ml						
10	pesticides mixture	4-8913	2mg/ml	neat 0.5ml						
11	PCBs	4-8246	0.5mg/ml	neat 2ml						
12	aromatic hydrocarbons	4-8743	0.2mg/ml	neat 5ml						
13	caffeine	C6035	1mg/ml	neat 1ml						
14	amitriptyline	A8535	1mg/ml	neat 1ml						
15	andrographolide	36,564-5	-	100mg 0.5ml						
16	androstane diol	A7755	-	100mg 0.5ml						
17	anisomycin	A9789	-	100mg 0.5ml						
18	atropine	A3661	1mg/ml	neat 1ml						
19	7-benzyloxytryptamine	B2001	-	100mg 0.5ml						
20	betamethasone	B7005	-	100mg 0.5ml						
21	betahistine	B4638	-	100mg 0.5ml						
22	8-bromoguanine	B7776	-	100mg 0.5ml						
23	8-bromooctanol	B5516	-	100mg 0.5ml						
24	cephalosporin	C3270	-	100mg 0.5ml						
25	6-chloro nicotinic acid	C1518	-	100mg 0.5ml						
26	5-hydroxytryptamine	H7752	-	100mg 0.5ml						
27	pentoxifyline	P1784	-	100mg 0.5ml						
28	uric acid	U2875	-	100mg 0.5ml						
29	epinephrine	E4250	-	100mg 0.5ml						
30	vanillin	V2375	-	100mg 0.5ml						

Table 1 (cont.) Contents of ligand cocktail

No.	Substance	Sigma Cat No.	Concn.	50ml DMSO		Submixtures						
				50ml Mix		SM1	SM2	SM4	SM8	SM16	SM32	
31	verapamil	V4629	-	100mg	0.5ml							
32	dopamine	H8502	-	100mg	0.5ml							
33	FMLP	F2009	-	100mg	0.5ml							
34	histamine	H7250	-	100mg	0.5ml							
35	melatonin	M5250	-	100mg	0.5ml							
36	lidocaine	L1138	1mg/ml	neat	1ml							
37	GABA	A2129	-	100mg	0.5ml							
38	met-enkephalin	M6638	-	100mg	0.5ml							
39	1,10-phenanthroline	P9375	-	100mg	0.5ml							
40	adenosine	A9251	-	100mg	0.5ml							
41	tetrahydrocannabinol	T4889	1mg/ml	neat	1ml							
42	acetylcholine	A6625	-	100mg	0.5ml							
43	mescaline	M5135	1mg/ml	neat	1ml							
44	morphine	M9524	1mg/ml	neat	1ml							
45	cocaine	C1528	1mg/ml	neat	1ml							
46	bromazepam	B5402	1mg/ml	neat	1ml							
47	benzocaine	B7150	1mg/ml	neat	1ml							
48	quinic acid	Q0500	-	100mg	0.5ml							
49	aspirin	A5376	-	100mg	0.5ml							
50	paracetamol	A5000	-	100mg	0.5ml							
51	indole acetic acid	I2886	-	100mg	0.5ml							
52	esculetin	E2631	-	100mg	0.5ml							
53	kinetin	K0753	-	100mg	0.5ml							
54	apricot oil	A7889	-	0.1ml	0.5ml							
55	bergamot oil	B4383	-	0.1ml	0.5ml							
56	cinammon oil	C7267	-	0.1ml	0.5ml							
57	bay oil	B4258	-	0.1ml	0.5ml							
58	aspartame	A5139	-	100mg	0.5ml							
59	thymol	T0501	-	100mg	0.5ml							
60	cineole	C8144	-	100mg	0.5ml							
61	saccharin	S1002	-	100mg	0.5ml							
62	acebutolol	A3669	-	100mg	0.5ml							
63	abrine	A5057	-	100mg	0.5ml							

Compounds were dissolved/dispersed in 50ml DMSO to give a stock solution

Ligand Mix comprised indicated volume of all 63 stock solutions made up to 50 ml with DMSO

The ligand mix was diluted 1/100 into top agar to give the final induction medium (~200ng/ml per ligand)

## Claims

1. A method for the preparation of cells which present on their surface a mutant of a natural G-protein coupled receptor (GPCR), said mutant being capable of binding a desired ligand which the natural receptor cannot, which method comprises:

- (a) generating a diverse collection of replicable expression plasmids which comprise (i) a coding sequence coding for a mutated form of a natural GPCR, wherein the mutation is of one or more amino acids in the transmembrane and/or extracellular domains of the natural GPCR, and (ii) expression control sequences operatively linked to said coding sequence to control expression of the mutant GPCR;
- (b) transforming cells of a host organism with said diverse collection of plasmids;
- (c) culturing the resultant collection of transformed cells for a sufficient time to permit expression of mutant GPCR from plasmids replicating within such cells;
- (d) contacting the thus cultured cells with the desired ligand; and
- (e) isolating cells wherein the surface presented mutant GPCR expressed by such cells binds to the desired ligand.

2. A method as claimed in claim 1 wherein the host organism cells transformed at step (b) are capable of expressing a recognisable phenotypic marker following signal transduction mediated by ligand binding to mutant GPCR expressed by a plasmid replicating within such cells, and at step (e) the cells isolated are those wherein the phenotypic marker is activated by binding of the desired ligand to mutant GPCR

expressed in such cells.

3. A method as claimed in claims 1 or 2 comprising the additional step of isolating from the cells isolated during step (e) the nucleic acid sequence coding for the said mutant GPCR, and repeating steps (a) - (e) using that sequence as the coding sequence referred to in step (a).
4. A method as claimed in claim 1, 2 or 3 wherein the said mutant GPCR is capable of binding a ligand which is substantially dissimilar in molecular shape and/or volume, type of bioactivity, pharmacophore, or chemical type to the ligand or ligands bound by the natural GPCR.
5. A method as claimed in claim 1, 2, 3 or 4 wherein random mutagenesis is used to generate the said diverse population of plasmids.
6. A method as claimed in any of claims 2 to 5 wherein the phenotypic marker is beta-galactosidase, recognisable by reaction with a chromogenic substrate therefor.
7. A method as claimed in any of claims 2 to 6 wherein the phenotypic marker is encoded by DNA in each plasmid of the diverse collection of plasmids.
8. A method as claimed in any of claims 2 to 6 wherein the phenotypic marker is encoded by DNA within the host organism other than DNA in a plasmid of the diverse collection of plasmids.
9. A method as claimed in any of the preceding claims wherein the host organism is a yeast.
10. A method as claimed in claim 9 wherein the yeast is *Saccharomyces cerevisiae*.
11. A method as claimed in any of the preceding claims wherein the natural GPCR

is a yeast mating factor receptor.

12. A method as claimed in claims 11 wherein the yeast mating factor receptor is Ste2.

13. A method as claimed in any of claims 1 to 10 wherein the natural GPCR is an odour receptor.

14. A method as claimed in any of the preceding claims comprising the additional step of isolating the mutant GPCR from the isolated cells.

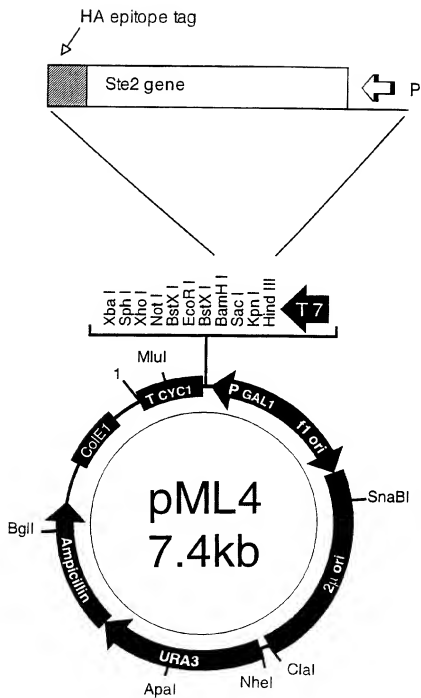
15. A mutant GPCR whenever prepared by a method as claimed in any of claims 1 to 14.

16. Nucleic acid coding for a GPCR as claimed in claims 3 or 14.

17. A diverse library of cells presenting on their surfaces mutants of a natural GPCR.

18. A library of cells comprising cells which present on their surface a mutant of a natural GPCR and cells which present the natural GPCR.

Fig 1/1



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB 97/00746

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K14/395 G01N33/50 G01N33/566 C12N1/19  
//C12N5/10, (C12N1/19, C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BIOLOGICAL CHEMISTRY 269 (12). 1994. 8831-8841. ISSN: 0021-9258, XP002037729 CLARK C D ET AL: "Systematic mutagenesis of the yeast mating pheromone receptor third intracellular loop." cited in the application see the whole document ---	1-18
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 90 (21). 1993. 9921-9925. ISSN: 0027-8424, XP002037730 BOONE C ET AL: "Mutations that alter the third cytoplasmic loop of the a-factor receptor lead to a constitutive and hypersensitive phenotype." see the whole document ---	1-18

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \* "A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

14 August 1997

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

Information publication No

PCT/GB 97/00746

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOLECULAR AND CELLULAR BIOLOGY 14 (5). 1994. 3339-3349. ISSN: 0270-7306, XP002037731 STEFAN C J ET AL: "The third cytoplasmic loop of a yeast G - protein - coupled receptors controls pathway activation, ligand discrimination, and receptor internalization." see the whole document ---	1-18
Y	WO 96 00739 A (MERCK & CO INC ;STRADER CATHERINE D (US); RIOS CANDELORE MARIA LUI) 11 January 1996 see the whole document ---	1-18
Y	WO 95 21925 A (AMERICAN CYANAMID CO ;PAUSCH MARK HENRY (US); OZENBERGER BRADLEY A) 17 August 1995 see example 11 ---	1-18
P,Y	MOLECULAR AND CELLULAR BIOLOGY 16 (9). 1996. 4818-4823. ISSN: 0270-7306, XP002037733 BUKUSOGLU G ET AL: "Agonist-specific conformational changes in the yeast alpha-factor pheromone receptor." see the whole document ---	1-18
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 93 (13). 1996. 6764-6769. ISSN: 0027-8424, XP002037734 KONOPKA J B ET AL: "Mutation of Pro-258 in transmembrane domain 6 constitutively activates the G protein-coupled alpha-factor receptor." see the whole document ---	1-18
P,X	JOURNAL OF MOLECULAR BIOLOGY 266 (3). 1997. 559-575. ISSN: 0022-2836, XP002037736 SOMMERS C M ET AL: "Genetic interactions among the transmembrane segments of the G protein coupled receptor encoded by the yeast STE2 gene." see the whole document ---	1-18
A	MOLECULAR AND CELLULAR BIOLOGY 15 (11). 1995. 6188-6195. ISSN: 0270-7306, XP002037732 PRICE L A ET AL: "Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway." see the whole document --- -/-	

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/GB 97/00746

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE BIOCHEMICAL JOURNAL, vol. 283, 1992, pages 1-19, XP002037739 T.M. SAVARESE ET AL.: "In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors" cited in the application see the whole document ---</p>	
A	<p>THE EMBO JOURNAL, vol. 4, no. 10, 1995, pages 2643-2648, XP002037740 N. NAKAYAMA ET AL.: "Nucleotide sequences of STE2 and STE3, cell type-specific sterile genes from Saccharomyces cerevisiae" cited in the application see the whole document -----</p>	

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB 97/00746

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9600739 A	11-01-96	CA 2193809 A EP 0770094 A	11-01-96 02-05-97
WO 9521925 A	17-08-95	AU 1846995 A CA 2183166 A EP 0745130 A	29-08-95 17-08-95 04-12-96